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Experimental Murine Candidiasis: Cell-Mediated Immunity After Cutaneous Challenge

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Attempts were made to isolate an antigen(s) from *Candida albicans* suitable for detecting delayed hypersensitivity in a murine model of candidiasis. Using footpad reactivity in cutaneously infected animals as the assay, comparisons were made of two commercial extracts and cell wall and cytoplasmic preparations made in the laboratory. An extract of the cell wall, a glycoprotein (GP) removed with ethylenediamine, and an extract prepared from the membrane fraction of disrupted *C. albicans* blastospores proved most useful in demonstrating delayed hypersensitivity in the murine model. The activity of the GP fraction was considerably reduced by oxidation with periodate and was abrogated entirely by digestion with proteolytic enzymes. The extract from the membrane fraction was obtained by incubating the insoluble membrane fraction with phosphate-buffered saline, pH 7.4, at 50°C, and the proteins in the extract were subsequently precipitated with ammonium sulfate to yield a test preparation that was approximately 75% protein and 25% carbohydrate. The precipitated extract was designated ppt-HEX. Footpad reactivity to ppt-HEX could be transferred with cells and not with serum if the cells were taken from animals at the appropriate time after sensitization. Since the membrane and GP fractions appear to elicit true delayed hypersensitivity reactions, further investigations into their specificity and biochemistry seem warranted.

Cell-mediated immunity (CMI) responses, primarily in the form of skin tests or in vitro assays of CMI, are frequently used to assess the immune status of a patient (32). Since a large percentage of the human population is reactive to *Candida albicans* antigens in one or more of the assays for CMI (5, 22), tests for reactivity to *Candida* are included in the battery of antigens used. A commercial preparation is commonly used for such tests, but little is known of its composition, and its activities have not been studied systematically under experimental conditions. Moreover, the preparation is preserved with phenol, so that it must be dialyzed before use in in vitro assays, and standardization of conditions for in vitro assays has been a problem. At least two investigators have attempted to standardize the conditions (1, 16), but others have simply used one preparation for skin testing and a different preparation for in vitro assays (4, 30).

Although commercial preparations have been used extensively in humans or with human cells, investigators using experimental animals have frequently prepared their own antigenic mixtures (2, 14, 15, 17, 20, 21, 27, 29, 31, 33, 36, 37) and have only infrequently used the commercial preparations (19). In this laboratory we were investigating a murine model of experimental candidiasis, and in the course of the investigations wanted to assay for delayed hypersensitivity but were uncertain of the most appropriate antigen preparation to use. We describe here the results of our search for an appropriate preparation, including assays with commercial extracts, extracts prepared as described in the literature, and extracts whose preparations were unique to this laboratory.

MATERIALS AND METHODS

Culture methods. *C. albicans* B311, originally obtained from H. Hasenclever, was used throughout these studies. It was maintained at 4°C by monthly transfer on Sabouraud dextrose agar.

Viable blastospores for inoculation into mice were grown in soy dialysate broth (28) for 18 h at 37°C on a gyratory shaker operating at approximately 165 rpm. Blastospores were harvested by centrifugation, washed three times with sterile nonpyrogenic saline (NPS) (Cutter Laboratories, Inc., Berkeley, Calif.), and counted in a hemocytometer before suspension in NPS at the appropriate concentration. Each suspension was further diluted and spread onto Sabouraud

agar plates to determine the viable count. The viable counts were always ±10% of the hemocytometer counts.

Viable blastospores from which antigens were prepared were inoculated in soy dialysate broth as above, but after harvest were washed three times with a tris(hydroxymethyl)aminomethane buffer (pH 7.2) containing 0.01 M $MgCl_2$ and stored at −20°C in the same buffer.

Fractionation procedures and source of test preparations. Precise details for the homogenization of the blastospores and for the sequence of centrifugations followed to obtain subcellular fractions rich in cell walls, membranes, mitochondria, and cell wall fragments (MM), ribosomes (RR), and soluble cytoplasmic substances (SCS) have been described elsewhere (12, 13). The RR and SCS fractions were used as test antigens without additional manipulation. The cell wall and MM preparations were extracted, however, and the various extracts were used as test antigens.

The cell wall fraction, i.e., that fraction which sedimented during the first centrifugation (400 × *g*), was extracted by two different methods. One portion of the walls was washed with saline and distilled water, lyophilized, and lipid extracted, and the lipid-extracted material was treated with ethylenediamine. Specific details are described elsewhere (13). The glycoprotein fraction (GP) resulting from this procedure was used as such, but portions were also oxidized with periodate (33) or incubated with papain (Sigma Chemical Co.) (25) or ficin (3). The papain used in this incubation was known to be free of glucanase and glycosidase activity (10). Incubations were performed in an acetate ethylenediaminetetraacetic acid-2-mercaptoethanol buffer (11). For the papain incubation, freshly prepared L-cysteine was added to the buffer (25). Ten milligrams of the GP was incubated for 4 h at 37°C in 2 ml of the buffer containing enzyme. Each mixture was then passed through a Bio-Gel P-60 column (25). The carbohydrate peaks resulting from the enzyme digestions and the protein peak from the oxidation procedures were pooled, dialyzed, and lyophilized. Each preparation was analyzed for protein by the method of Lowry et al. (23), using a bovine serum albumin standard, and for total carbohydrate by the orcinol-sulfuric acid method (34), using a mannose standard. Each preparation was analyzed for its monosaccharide content by gas-liquid chromatography (GLC) (13) and by thin-layer chromatography (TLC) on silica gel (26). For the GLC and TLC assays, the substrate was hydrolyzed with 1 N HCl in methanol before analysis (13).

A second portion of the cell walls was washed with sodium dodecyl sulfate, extracted with dilute alkali, and treated sonically exactly as described by Reiss et al. (27). This procedure results in three fractions, designated peptidoglucomannan (PGM), soluble mannoglucan (sMG), and insoluble mannoglucan. The insoluble mannoglucan was not used in any way.

The MM fraction was treated in the following manner. One gram of lyophilized MM was suspended in 100 ml of phosphate-buffered saline, pH 7.4, and extracted at 50°C for 1 h with intermittent swirling. The residue was removed by centrifugation at 20,000 × *g* for 20 min, and the supernatant was cooled in an ice bath. Initially, such supernatants were dialyzed against NPS; the protein content was estimated by the method of Lowry et al. (23) and used as such as a footpad-testing reagent. This preparation will be referred to later as crude-HEX. In later experiments, the protein was precipitated from the supernatant by the addition of solid ammonium sulfate to equal 100% saturation. After 30 min on ice with constant agitation, the precipitate was sedimented by centrifugation at 20,000 × *g* for 30 min, dissolved in 20 ml of sterile NPS, and dialyzed at 4°C against 200 volumes of sterile NPS for 48 h with six changes. The protein content was determined by the method of Lowry et al., (23), the solution was adjusted to the desired concentration with NPS, and the extract was stored at −20°C in 2-ml portions. This extract was designated ppt-HEX.

Two commercial preparations were tested along with those prepared in the laboratory. Dermatophytin O, undiluted and preserved with 0.45% phenol, was obtained from Hollister-Stier Laboratories, Spokane, Wash., and *Candida* (*Monilia*) *albicans* allergenic extract, 40,000 protein nitrogen units per ml, preserved with 0.4% phenol, was obtained from Greer Laboratories, Lenoir, N.C.

Animal methods. CBA/J mice (Jackson Laboratories, Bar Harbor, Me.), approximately 12 weeks old at the beginning of experiments, were used throughout these studies. They were numbered by ear punch and randomized into groups of 10 to 15. Mice were inoculated once or twice cutaneously into the shaved flank with 1.0×10^6 viable *C. albicans* blastospores suspended in 0.05 ml of NPS. When inoculated twice, the interval between the first and second inoculations was 2 weeks. Further, some animals infected cutaneously and footpad tested were challenged intravenously with 1.0×10^4 or 5.0×10^4 viable blastospores suspended in 0.5 ml of NPS.

Footpad testing was accomplished by the injection, with a micrometer syringe, of 0.02 ml of test preparation suspended in NPS. Each foot was measured with a Schnelltäster calipers (H. Kröplein GmbH, Schluchtern, West Germany) before and at selected intervals after the injection of antigen. The mean net increase in footpad thickness for each group was determined after the subtraction of pre- and postinjection values. In each experiment the animals were randomized, coded, injected by one individual, and measured by a second individual to whom the code was unknown. At the time of footpad testing, animals were housed in a random manner as well, so that one group representing one experimental condition was not housed in a single cage.

Peritoneal exudate cells used for passive transfer experiments were obtained from mice stimulated with casein 48 h before harvest (24). Immediately before sacrifice all animals were bled to obtain serum. The ratio of donors to recipients was 2:1; i.e., the peritoneal exudate cells harvested from two mice were pooled and transferred to one recipient, and the sera were pooled as well, each serum recipient receiving approximately 0.5 ml of serum pooled from two donors. In all experiments, cells and serum were administered to the recipients intravenously. Footpad testing was done 18

h after transfer. A small portion of each serum sample was tested for antibodies to ppt-HEX (2 mg/ml) and SCS (5 mg/ml) by counterimmunoelectrophoresis (D. K. Giger, J. E. Domer, and J. T. McQuilty, Jr., manuscript in preparation).

RESULTS

Survey of cell wall and cell sap antigens. Mice that had been infected cutaneously twice, 2 weeks apart, were footpad tested 2 weeks after the second infection with three cell wall fractions, GP, PGM, and sMG, and three cell sap preparations, MM, RR, and SCS. Separate groups of uninfected mice were footpad tested with the same preparations to test the nonspecific inflammatory capacity of the antigen. Each fraction was adjusted to 20 μg by weight per test dose, with the exception of the RR, which was adjusted to 20 μg of protein per dose. The data are summarized in Fig. 1. Reactions were measured 4, 24, and 48 h after injection of the test preparation.

Of the cell wall fractions, the PGM elicited the greatest responses 24 h after injection. However, in our hands PGM was poorly soluble, the data were not reproducible from experiment to experiment, and PGM elicited reactions at 4 h that were often larger than the 24-h reactions, making the 24-h reactions difficult to interpret. Similar difficulties were experienced with the sMG as well. The GP also elicited large early reactions (see the 4-h responses to GP on Fig. 1) that appeared to be nonspecific, since similar reactions occurred in uninfected animals, but it was soluble and in previous experiments had been shown to elicit greater 24-h responses, so it was retained for further testing (see below).

Of the non-cell wall preparations tested, only the MM, that fraction consisting of membranes, mitochondria, and cell wall fragments, elicited substantial 24-h responses. The RR fraction elicited a poor response, and SCS elicited no responses detectable 24 to 48 h after testing. There were, however, two problems with the MM fraction. First, it was insoluble and could possibly

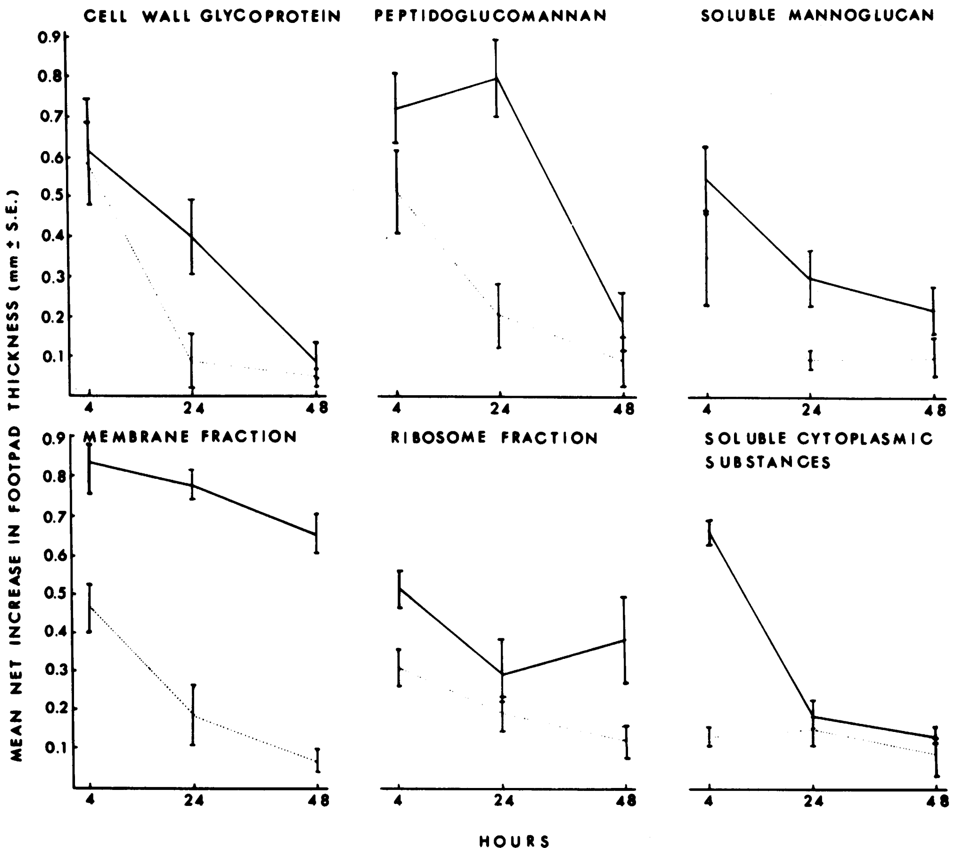

FIG. 1. *Mean net footpad responses in uninfected animals (· · · · ·) and in animals infected cutaneously with viable C. albicans blastospores (——) 2 weeks before footpad testing with cell wall and cytoplasmic fractions of Candida.*

elicit in situ antibody formation (6, 7); second, it also elicited large 4-h reactions. Efforts, therefore, were directed toward obtaining a soluble fraction of the MM preparation with reduced activity in terms of eliciting early reactions.

Studies with extracted MM and comparison with commercial preparations. The MM fraction was extracted with hot buffer as described above, and the crude extract was tested, along with the Greer and Hollister-Stier antigens, in mice infected once or twice with viable *Candida*. The experimental design, summarized in Table 1, included animals that had been infected once, 13 days (group I), or 28 days (group III) before testing, animals that had been infected twice and footpad tested twice (group I), i.e., 13 days after each cutaneous inoculation, animals that had been infected twice but footpad tested only once (group II), i.e., 13 days after the second cutaneous inoculation, and animals that had never been infected (group IV). Group I is referred to at two places above because those animals served two functions in the experiments: to detect responses induced by a single inoculation 2 weeks before footpad testing, and to determine the effect of footpad testing before the second cutaneous infection on a subsequent footpad test. The Greer and Hollister-Stier antigens were tested at 1:10, 1:40, and 1:160 and at 1:8, 1:32, and 1:128 dilutions, respectively. The crude-HEX was tested at 5, 20, and 80 μg of protein per dose. The most dilute of the commercial antigens elicited no responses, and the most concentrated elicited large reactions in uninfected animals. The differences between uninfected and infected animals were no greater that those observed with the intermediate dilution of either commercial antigen. Therefore, only the data for the 1:40 and 1:32 dilutions of Greer and Hollister-Stier are presented in Fig. 2. When the crude-HEX was concentrated to 4 mg of protein per ml, the concentration necessary to achieve a test dose of 80 μg, a precipitate formed which apparently contained the active ingredient because the supernatant no longer elicited positive reactions. Five micrograms of crude-HEX protein elicited reactions that were less intense at all observation times than those elicited by 20 μg. Therefore, only those data derived from testing with 20 μg of protein per dose are presented in Fig. 2. In these studies the observation period was expanded to include 4-, 7-, 24-, and 48-h measurements.

TABLE 1. *Summary of experimental design for comparing crude-HEX and the Greer and Hollister-Stier antigens*

Day	Group				
	I	II	III	IV	V
0	i.c.[a]	i.c.	i.c.		
13	Footpad tested				
15	i.c.	i.c.			
28	Footpad tested	Footpad tested	Footpad tested	Footpad tested	
30	i.v.[b]	i.v.	i.v.	i.v.	i.v.

[a] Intracutaneous infection with 1.0×10^6 viable *C. albicans* blastospores.

[b] Intravenous challenge with 1.0×10^4 or 5.0×10^4 viable *C. albicans* blastospores.

The following comments are based on the data presented in Fig. 2. The Greer and Hollister-Stier preparations did not elicit positive 24-h responses in mice infected cutaneously only once, 13 or 28 days before testing, whereas the crude-HEX preparation elicited positive but minimal reactions in similar animals. A second cutaneous inoculation boosted the level of hypersensitivity of the animals so that all three test preparations elicited positive 24-h reactions 13 days after the second cutaneous inoculation. If, however, animals infected for a second time had been footpad tested 2 days before the second infection, their 24-h reactions 13 days after the second infection were almost always less than those in animals infected twice cutaneously but not footpad tested 13 days after the first infection. Although the differences were only statistically significant in one of three experiments, the same relationship between the two groups held true in each experiment.

In summary, crude-HEX appeared more sensitive than either of the commercial preparations in that it detected hypersensitivity in mice infected only once, whereas the Greer and Hollister-Stier preparations did not detect low levels of hypersensitivity. However, the crude-HEX preparation continued to elicit strong early reactions, i.e., at 4 and 7 h, which clouded the interpretation of the 24-h reaction.

Since antibodies directed to the carbohydrate in the crude-HEX preparation could have been responsible for the large early reactions observed in both infected and uninfected animals (the crude-HEX was approximately 35% carbohydrate), the proteins of the crude-HEX preparation were precipitated with ammonium sulfate. Mice infected once or twice cutaneously before footpad testing, as well as uninfected mice, were footpad tested with the ppt-HEX adjusted to 20 μg of protein per test dose. In this experiment, no animals were footpad tested twice. The specific data for this experiment are not presented because they were very similar to the data obtained in a subsequent, somewhat expanded experiment presented in Fig. 3. Additionally, in these and subsequent experiments, the footpads were measured 15 min after the injection of

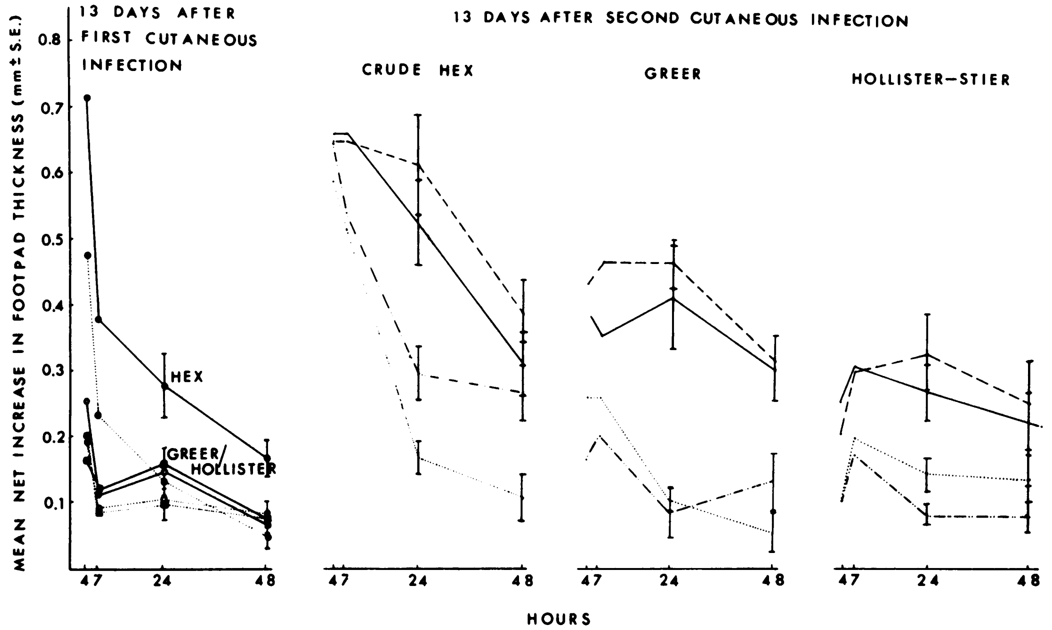

FIG. 2. *Mean net footpad responses to three antigen preparations in uninfected animals (· · · · ·), in animals infected once 13 days before testing (first frame), in animals infected once 28 days before testing (- · · -), and in animals infected twice and footpad tested once (- - -) or twice (——).*

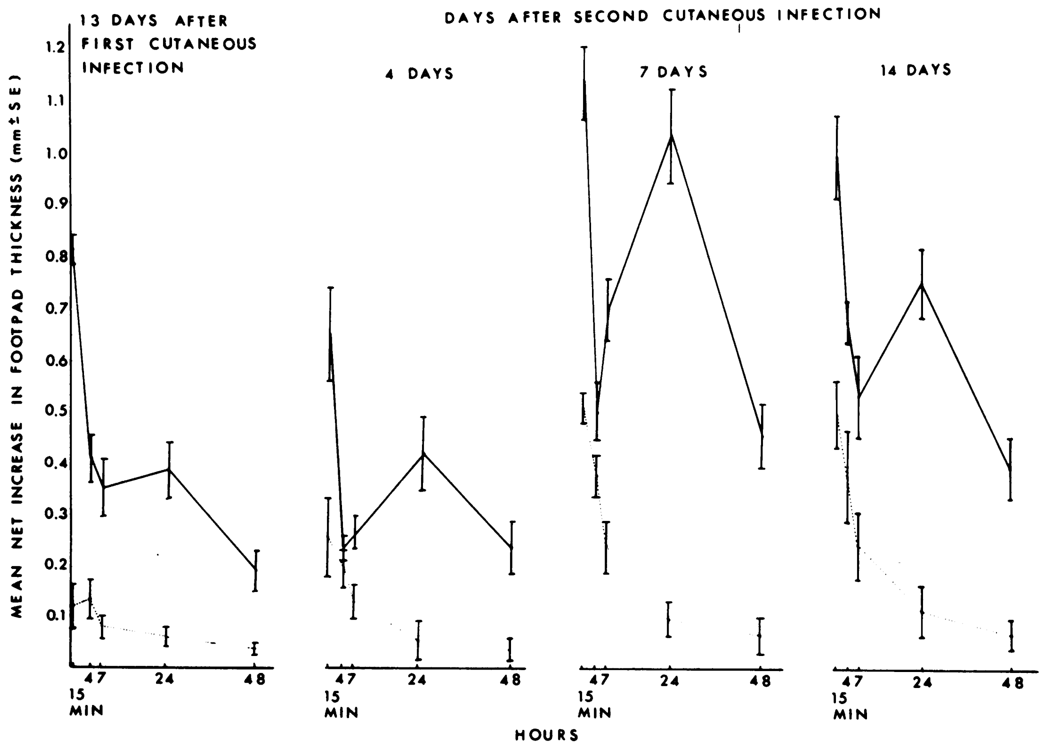

FIG. 3. *Mean net footpad responses to ppt-HEX in uninfected animals (· · · · ·) and in infected animals (——) at selected intervals after one or two cutaneous inoculations of viable C. albicans blastospores.*

antigen as well as at 4, 7, 24, and 48 h. Ammonium sulfate precipitation of the crude-HEX resulted in an extract that did not elicit 4- and 7-h reactions of the magnitude of the crude preparation; in fact, the 24-h responses in animals infected twice cutaneously were maximum when compared with the 7-h response (see Fig. 3, fourth frame). Although the reactions were all well above control levels in animals infected once cutaneously 13 days before testing, the 7- and 24-h reactions were of a similar magnitude. The data in Fig. 3 are the result of an experiment designed to investigate the possibility that a maximum level of delayed hypersensitivity occurred earlier than 14 days after the second cutaneous inoculation. Five groups of mice were infected cutaneously on day zero, and five groups were held in reserve as uninfected controls. On day 13, one group each of the infected and uninfected animals was footpad tested with ppt-HEX, 20 μg of protein per dose. On day 14, the other four groups of previously infected mice were infected cutaneously a second time, and footpad tests were performed 4, 7, 11, and 14 days thereafter, each time using a fresh group of infected and uninfected animals. These are the data that are presented in Fig. 3. The 11-day observations are not presented since they were similar to those observed at 14 days. On the basis of these data, then, there appears to be a peak of delayed hypersensitivity 7 days after a second cutaneous inoculation of viable *Candida*, and the hypersensitivity wanes considerably by 14 days.

Since we felt that it was still possible that the 24-h reaction to ppt-HEX in sensitized animals could be due to a late arthus reaction, attempts were made to transfer the hypersensitivity with cells and serum from animals infected twice cutaneously. The first such experiment was with cells and serum taken from animals 14 days after the second cutaneous infection. The total number of recipients per group was 10. There was a slightly elevated 15-min reaction in serum recipients in this experiment, but neither serum recipients nor cell recipients developed reactions significantly different from those of controls (data not shown). Subsequently, two more attempts were made to transfer the reactivity, but this time with cells and serum taken from mice 7 days after the second cutaneous inoculation (Fig. 4). In experiment I, cells and serum were taken from infected animals only and transferred to naive recipients. In experiment II, control, uninfected mice also served as donors of cells and serum. The 24-h reaction demonstrable with the ppt-HEX in infected animals was only transferable with cells from infected animals. In the first experiment, some early (15 min) reactivity

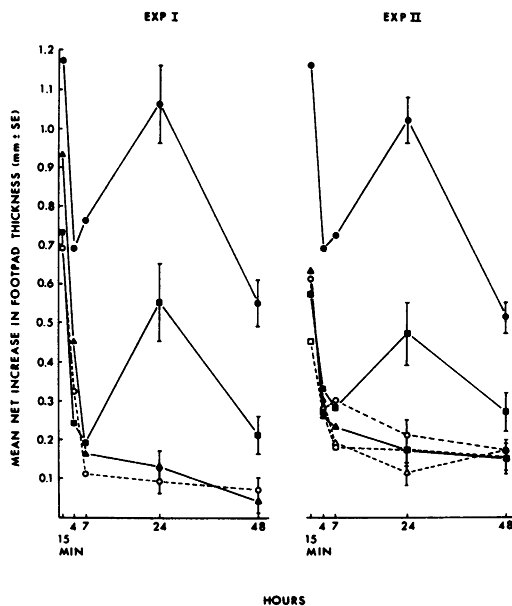

FIG. 4. *Mean net footpad responses to ppt-HEX in uninfected control animals* (○), *in infected animals 7 days after a second infection with viable C. albicans blastospores* (●), *and in animals receiving peritoneal exudate cells from uninfected* (□) *or infected* (■) *animals, or serum from uninfected* (△) *or infected* (▲) *animals.*

was transferred with serum, but there was no difference between serum recipients and control animals in the second experiment. The lack of an early response may have been due to the fact that no antibodies to ppt-HEX were demonstrable in the transferred sera. The same sera were also negative with SCS.

Further experiments with GP. After learning in the above experiments with ppt-HEX that a maximum delayed hypersensitivity response occurred 7 days after a second cutaneous inoculation with viable blastospores, we decided to test GP at that interval and to determine whether the 24-h response to GP could be abrogated by periodate oxidation and/or digestion with proteolytic enzymes. Chemical analyses of modified and unmodified GP, as well as of the ppt-HEX, are summarized in the next section. Separate groups of infected and uninfected mice were footpad tested with 30 μg of unmodified GP and with unmodified, oxidized, and ficin- and papain-digested GP sieved through Bio-Gel P-60 (Fig. 5). Periodate treatment resulted in a significant reduction in activity of GP, but the 24-h responses were still significantly greater than control reactions. On the other hand, papain or ficin digestion (ficin data not shown) completely abrogated the ability of the GP to

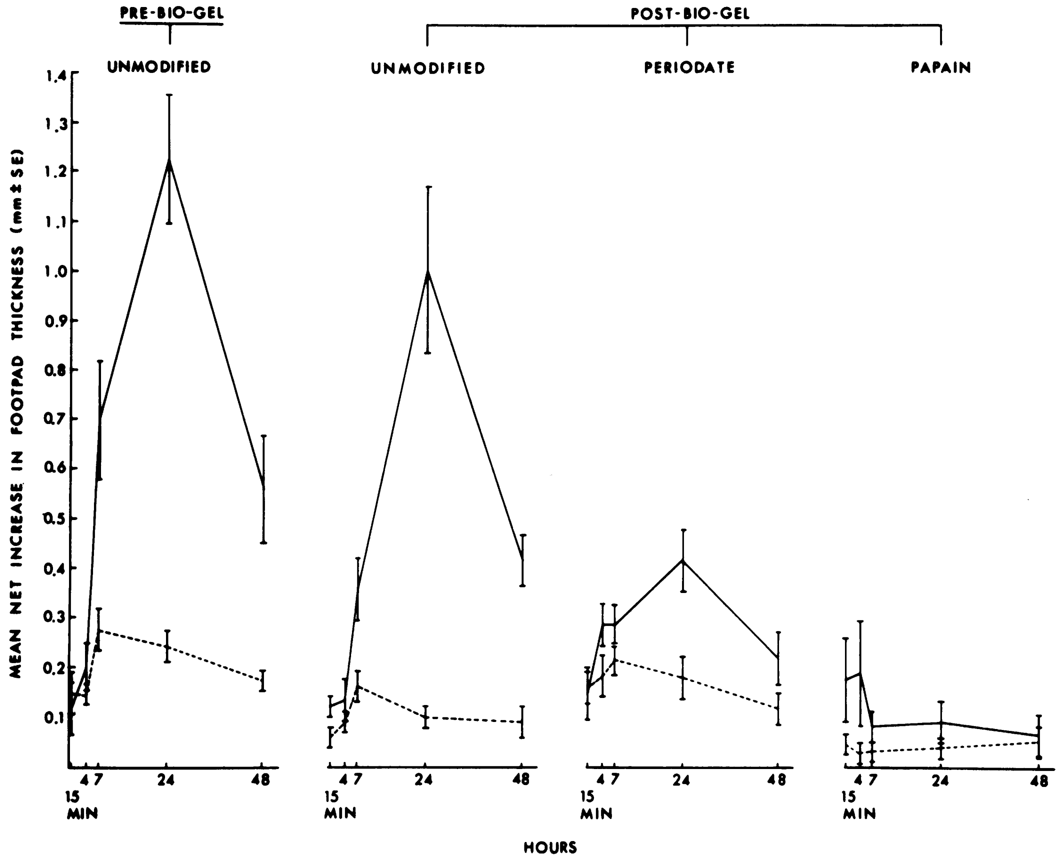

FIG. 5. *Mean net footpad responses to various preparations of cell wall glycoprotein (GP) in uninfected animals* (· · · · ·) *or in animals 7 days after a second cutaneous inoculation with C. albicans blastospores* (——).

elicit a positive delayed reaction. These data would suggest that the protein portion of the preparation is vital to the elicitation of a delayed response to the GP. Further, the reaction could, in fact, be due to a glycoprotein that was partially oxidized, resulting in the decreased responses observed with the GP oxidized with periodate.

Chemical analyses of ppt-HEX and GP. The carbohydrate and protein contents of ppt-HEX and unmodified GP, as well as of oxidized and papain-digested GP, are presented in Table 2. The ppt-HEX preparation was predominantly protein, whereas the GP preparation was predominantly carbohydrate. Moreover, the predominant sugar in the GP was mannose, whereas there was more glucose in the ppt-HEX. Periodate treatment did not result in the complete oxidation of the GP, but did result in a significant decrease in carbohydrate reactive material. There was not a good correlation between the orcinol and GLC data, especially with the periodate-treated GP. We believe that the GLC data represent a more accurate estimate of the carbohydrate material, however, because when the hydrolysate of the periodate-treated GP was chromatographed by TLC alongside a hydrolysate prepared from an equal weight of unmodified GP, the monosaccharides were barely visible in the periodate-treated preparation. Visually then, there appeared to be considerably more than a 50 to 60% reduction in carbohydrate. Papain treatment resulted in a considerable reduction of the proteinaceous portion of the GP, although there was still a small percentage of reactive material remaining.

Intravenous challenge. We have shown elsewhere (Giger et al., in preparation) that animals infected cutaneously once or twice with viable *Candida* develop varying degrees of resistance to reinfection depending on the dose of *Candida* and number and timing of cutaneous infections. We decided, therefore, to challenge the animals being used in the footpad-testing experiments to determine whether footpad testing influenced the degree of resistance. All ani-

mals were challenged intravenously 2 days after footpad testing. Those footpad tested with the Greer and Hollister-Stier antigens were challenged with 1.0 × 10^4 viable blastospores, whereas those footpad tested with crude HEX were challenged with 5.0 × 10^4. Life table analysis of the data 50 days after intravenous challenge is presented in Table 3. Data from animals infected once or twice cutaneously but never footpad tested are not presented in the table

TABLE 2. *Protein and carbohydrate content of ppt-HEX, unmodified GP, and GP treated in several ways*

Fraction	Total carbohydrate		Mannose/glucose[a]	Total protein[b] (%)	Protein/carbohydrate[c]
	Orcinol[d] (%)	GLC[e] (%)			
ppt-HEX	25	14	1:2	75	3.8:1
Glycoprotein					
Pre-Bio-Gel	73	54	4:1	16	1:4.0
Post-Bio-Gel	69	ND[f]	6:1	13	1:5.3
Periodate	31	8	3:1	24	1:0.8
Papain	84	93	8:1	3	1:29.5

[a] Ratios based on GLC analysis.
[b] Method of Lowry et al. (23).
[c] Based on average of orcinol and GLC data.
[d] Method of Svennerholm (34), using mannose as standard.
[e] Chromatography of trimethylsilyl ether derivatives (13), using dulcitol as internal standard.
[f] Not done quantitatively.

TABLE 3. *Life table analysis (9) after intravenous challenge with viable Candida blastospores of previously uninfected and cutaneously infected mice footpad tested with varying doses of antigen*

Group[a]	Crude HEX (5 × 10^4)[b]		Greer (1 × 10^4)		Hollister-Stier (1 × 10^4)	
	n	% Survival ± SE[c]	*n*	% Survival ± SE	*n*	% Survival ± SE
V (challenge controls)	20	0	20	30 ± 10	19	16 ± 8
IV (footpad test controls)						
L	10	0	10	40 ± 16	10	0
I	10	0	10	30 ± 14	10	60 ± 16
H	10	20 ± 13	10	30 ± 14	10	33 ± 16
Combined		7 ± 5		34 ± 18		31 ± 18
III (one infection)						
L	10	20 ± 13	10	10 ± 9	10	10 ± 9
I	10	20 ± 13	10	30 ± 13	10	30 ± 15
H	10	10 ± 10	10	60 ± 15	10	40 ± 15
Combined		17 ± 7		30 ± 18		26 ± 16
II (two infections, one test)						
L	15	27 ± 12	15	13 ± 9	15	74 ± 11
I	15	40 ± 13	15	67 ± 12	15	74 ± 11
H	15	40 ± 13	14	72 ± 12	15	93 ± 7
Combined		36 ± 7		50 ± 16		80 ± 12
I (two infections, two tests)						
L	15	40 ± 13	15	60 ± 13	15	100
I	15	27 ± 12	15	46 ± 13	15	93 ± 7
H	15	27 ± 12	14	93 ± 7	14	79 ± 11
Combined		31 ± 7		66 ± 14		91 ± 8

[a] Group numbers used here correspond to those presented in Table 1. L, I, and H indicate low, intermediate, and high doses of antigen used for footpad testing; for crude-HEX, it corresponded to 5, 20, and 80 μg of protein per footpad dose; for the Greer antigen, dilutions of 1:10, 1:40, and 1:160; and for the Hollister-Stier antigen, 1:8, 1:32, and 1:128.
[b] Dose of viable *C. albicans* blastospores administered.
[c] SE, Standard error of the mean.

since they have been presented elsewhere (Giger et al., in preparation).

Those animals infected twice cutaneously consistently showed the greatest level of resistance to reinfection, regardless of the challenge dose of blastospores. A few animals cutaneously infected only once, 4 weeks before challenge, showed resistance to reinfection, and 2 of the 10 animals footpad tested with the 80-μg dose survived to 50 days. By way of comparison, animals infected twice cutaneously but never footpad tested had a survival rate of 42% $\pm$ 11, whereas the uninfected controls had a survival rate of 5% $\pm$ 5 (Giger et al., in preparation). Footpad testing with any dose of crude-HEX, then, did not seem to change significantly the survival rates of animals when challenged with 5.0×10^4 blastospores. Cultures of the kidneys of surviving animals 50 days after intravenous challenge did not reveal any striking differences between groups. The kidneys of approximately 25% of those surviving beyond 50 days, however, were negative on culture.

The survival data obtained when the Greer and Hollister-Stier preparations were used as footpad-testing antigens would seem to indicate that footpad testing, particularly with the intermediate and higher concentrations of those antigens, favorably influenced survival after intravenous challenge. Perhaps the antigen serves to boost the immune response and afford added protection. For example, we reported previously that mice infected cutaneously once 4 weeks before intravenous challenge and never footpad tested did not develop resistance to reinfection if not footpad tested (Giger et al., in preparation), whereas animals treated in a similar manner in these experiments but footpad tested with the highest concentration of the commercial antigens just before intravenous challenge did seem to develop some resistance to reinfection. The same thing was true for animals receiving two infections. Animals that had not been footpad tested had survival rates of 60 and 54% in two experiments compared with 30 and 13% previously reported in uninfected challenge controls (Giger et al., in preparation). The booster effect of footpad testing in animals infected twice cutaneously before intravenous challenge was most obvious at the highest dose of antigen when testing with the Greer preparation, but all three doses of antigen seemed to boost resistance to reinfection in the animals tested with the Hollister-Stier antigen.

DISCUSSION

Two extracts of *C. albicans* have been described here which elicited delayed hypersensitivity in mice sensitized by infection: ppt-HEX and GP. The ppt-HEX was more sensitive than two commercial preparations with which it was compared, since it detected low levels of sensitivity when they did not. The ppt-HEX was predominantly protein, and reactivity to it demonstrable in the footpad assay was transferable with cells and not with serum. There is always the possibility that B lymphocytes in the preparation transferred the reactivity, but that seems unlikely for the following reasons. First, Crowle (8), in reviewing the conditions necessary for demonstrating delayed hypersensitivity in the mouse, pointed out that test antigens that were particulate and retained at the site for an extended period were more likely to elicit responses which could be "delayed" in onset but which were, in fact, due to B lymphocytes manufacturing antibody in situ. The ppt-HEX was soluble and unlikely to fit in that category. Secondly, North and Spitalny (24) indicated that the majority of lymphocytes accumulating in the peritoneal cavity after stimulation with casein were T cells. Moreover, if the reaction was mediated by antibody, serum should have transferred it, and the peak in vivo response should not have waned as quickly as it did. In the past (Giger et al., in preparation), animals treated in a similar manner developed few precipitins by 13 days after one cutaneous inoculation with viable *Candida* but had developed many precipitins by 13 days after the initiation of the second cutaneous infection, the time at which delayed hypersensitivity waned.

The footpad reactivity to GP, a glycoprotein extracted from cell walls with ethylenediamine, was reduced by periodate oxidation, but was abrogated entirely by a proteolytic enzyme, suggesting that the intact protein moiety was essential for the delayed reaction. It is possible that the intact glycoprotein was responsible for the activity, however, and that some activity was retained after oxidation simply because the oxidation was not complete. In fact, there is considerable conflict in the literature with respect to carbohydrate and protein components of fungal antigen preparations and which component is responsible for eliciting the delayed response. Suzuki and Hayashi (33) for example, claim to have induced and demonstrated delayed hypersensitivity to pure polysaccharide, a mannan extracted from a culture filtrate of *Candida*. The activity of the mannan was completely abolished by periodate oxidation. They did not test their extract in animals sensitized by infection, however, and they found it necessary to use Freund complete adjuvant when sensitizing guinea pigs to induce strong hypersensitivity. Reiss et al. (27) reported delayed hypersensitivity to mannan as well, but, unlike Suzuki and

Hayashi, they were unable to detect immediate-type responses to mannan. Contrary to both groups of investigators just mentioned, Aoki et al. (2) were unable to sensitize guinea pigs with polysaccharide alone, but found that a mixture of protein and crude polysaccharide administered in Freund complete adjuvant was able to induce delayed hypersensitivity. Further, they reported that Pronase treatment of the crude antigen preparation rendered it inactive in terms of the induction of delayed hypersensitivity and only weakly active in eliciting an established hypersensitivity. Most other investigators agree with the assessment that polysaccharide, specifically mannan of *C. albicans*, is largely responsible for immediate-type responses, and that protein is responsible for the delayed component of the reaction (2, 14, 15, 20, 21, 31, 35). Kabe et al. (20, 21), in fact, tested their extracts in asthmatic humans as well as sensitized guinea pigs to reach that conclusion. Moreover, they compared dermal and pulmonary sensitivities by performing skin and inhalation challenges, both of which correlated well with respect to immediate and delayed reactions.

An additional line of investigation that should follow from these studies is the possibility that the commercial preparations boost an immune response when used as footpad-testing antigens. It is difficult to determine precisely which arm of the immune system they boost, but based on sequential footpad assays in the same animal, it appears that they boost the humoral rather than the cellular arm. If such is, in fact, the case, this is an undesirable observation with respect to humans. If antibody levels can ever be shown to have diagnostic or prognostic value in candidiasis, it would be inappropriate to have a skin-testing antigen that could stimulate a secondary antibody response. The crude-HEX used in these studies did not appear to have that capacity, but that aspect of the work needs to be more thoroughly investigated before firm conclusions can be drawn.

In summary, both the GP and ppt-HEX appear worthy of consideration for future studies, including studies of specificity and studies designed to test the suitability of each preparation for in vitro assays of cell-mediated immunity. The glycoprotein, in fact, has been tested in lymphocyte transformation assays by others (18) and found to be considerably more potent than cytoplasmic antigens. Further, both antigens are relatively easy to obtain and, appear to be reproducible from batch to batch.

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